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# A Simple, Direct Method for Measurement of Microfibril Angle in Single Fibers Using Differential Interference Contrast Microscopy

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## ABSTRACT

Differential interference contrast (DIC) microscopy can image the cellulose microfibrils in the S2 layer of delignified secondary walled fibers. The utility of this simple microscopic method for measuring microfibril angle (MFA) was validated relative to x-ray diffraction and polarization confocal microscopy methods. The mean MFA measured by DIC microscopy was highly correlated, $R^2 = 0.96$, with MFAs measured by x-ray diffraction for both earlywood and latewood samples over a range of MFAs (5-50°). The mean and distribution of MFA in fiber populations measured with DIC microscopy were the same as those obtained with polarization confocal microscopy. This direct DIC method is readily used with a wide range of secondary walled fibers from softwoods, hardwoods, and nonwoody fibers.

## INTRODUCTION

The hygroexpansivity, strength, and elasticity of fibers determine much of the mechanical properties, including tensile and compressive strengths, bending stiffness and modulus of elasticity, as well as tangential and longitudinal shrinkage, of wood and paper materials [1-9]. Experimental and theoretical evidence clearly demonstrate that the tensile strength and stiffness of fibers are well correlated with the microfibril angle (MFA) in the S2 layer of plant cell secondary walls [7-9]. MFA is the angle at which cellulose microfibrils are aligned relative to the longitudinal axis of the fiber cells. Fibers with a low MFA, <10°, have high tensile strengths and high elastic moduli relative to fibers with high MFA. The importance of the cellulose MFA in the S2 layer of fiber cells has generated strong interest in developing methods to measure this critical fiber property using both wood and fiber samples.

A number of direct and indirect methods have been developed for measuring the MFA in the S2 layer of wood and fibers. Most indirect methods use simple microscopic methods to visualize some cell wall feature such as natural or induced cell wall striations [10] or the pit aperture angle [10] that are presumed to be coaligned with the cellulose microfibrils or some bound contrast agent such as iodine crystals [11], congo red [12], or a clear space left after hydrolysis of cellulose microfibrils with soft rot fungi [10,13]. Another indirect method uses x-ray diffraction [14-17]. Direct methods visualize the cellulose microfibrils with UV light and phase microscopes or polarized light [10,18-20]. Each method has its advantages and disadvantages, and no one method is clearly superior for all applications.

The most widely used methods to measure MFA are x-ray diffraction [6,17] and polarized light microscopy [10, 18-20]. Both of these methods exploit the crystalline nature of the cellulose microfibrils. X-ray diffraction has the benefit of being a very quick technique for measuring the mean MFA of large sample numbers and sizes [17]. X-ray diffraction is limited to use with wood

samples and cannot be used on dispersed pulped fiber samples since it requires that fibers are oriented parallel to one another. In addition, the distribution of individual fiber MFAs is lost in the average of the 002 equatorial reflection plane [14-16]. Polarized light microscopy methods determine the MFA of single fiber cells. Since it measures the MFA of single fiber cells, an advantage of polarized light microscopy is that it works with both wood and pulped fibers. The disadvantage is that it requires tedious sectioning and preparation or hazardous mercury impregnations to obtain single wall measurements, due to the fact that the MFA is opposite in orientation on the two sides of the cells [10, 17]. In addition, for thick-walled fibers such as southern pine, the S1 and S3 layers are thick enough to introduce significant error into the measurements made with traditional polarizing microscopes [20]. The more recently developed polarization confocal microscopy methods can also be used on wood and pulped fibers with less sensitivity to distortions from S1 and S3- layers [12, 19]. Since fibers are optically sectioned with this technique, most of the difficult sample preparations associated with polarized microscopes are not needed [12, 19]. The disadvantage to polarization confocal microscopy is the cost of the instrument. Few facilities have the appropriate equipment or capital to invest in such a highly specialized instrument.

We report here a direct method for measuring the MFA in single fibers using simple image analysis and Differential Interference Contrast (DIC) microscopy. We show that the cellulose microfibrils in the S2 layer of extensively delignified fibers are directly visible in DIC microscopes. DIC images captured with a digital camera and the angles of cellulose microfibrils are measured relative to the longitudinal axis of the fiber using standard image-analysis tools. We show that measurements of MFA with this direct DIC method correlate highly with those obtained with both x-ray and polarization confocal microscopy congo red methods. The principal advantages of DIC

microscopy are that it is simple, uses relatively inexpensive equipment, and works with a wide range of fibers from woody and nonwoody plants.

## EXPERIMENTAL

### Plant Fibers

Wood and pulp samples were from *Pinus taeda.*

### X-ray Diffraction

Wood samples were visually screened for compression wood and uniform MFA using standard x-ray diffraction analyses before and after pulping [6]. From these samples, three earlywood samples (E3, E7, and 7E3) and three latewood samples (L4, 20L2, and L5) of low (0-10°), medium (15-30°), and high (31-50°) MFA were selected. Their MFAs were confirmed in the tangential and radial positions using x-ray diffraction.

### Fiber Preparation from Wood or Pulp

Wood samples chosen for different MFAs were extensively delignified at 65-70°C overnight, using peracetic acid made up of 50% hydrogen peroxide (30%) and 50% glacial acetic acid. All wood samples were then carefully rinsed with $H_2O$, neutralized with 1M NaOH, and allowed to air dry overnight. The MFA in kraft pulp samples have also been determined, but in general the further delignifying fibers to low kappa numbers show higher numbers of fibers with visible microfibrils. Temporary wet slides were made from the dispersed fibers using a pipette to collect fibers and move them onto a glass slide. A needle was used to further spread the fibers to minimize overlap.

## DIC Microscopy, Image Capture, and Analysis

A Zeiss Axioplan 2 microscope was used, equipped with standard DIC optics that included a variable intensity halogen lamp, a polarizer, a Wollaston prism, analyzer, and mechanical stage. Observations were made using either a 20x Plan-Neofluor lens with a numerical aperture (NA) of 0.5 or a 40x Plan-Neofluor lens with a NA 0.75. Due to their higher NA, oil-immersion lenses perform better than non oil-immersion lenses for visualizing microfibrils, but oil tends to slow the overall image sampling procedure and was not used in this study. A Sony DKC 5000 digital camera was used to capture images onto a PC microcomputer. The protractor tool of the image analysis software from Scion Image was used manually to measure to the nearest fraction of a degree (two decimal places) the angle of 4 microfibrils per fiber image to obtain the average MFA for individual fibers. Data from Scion Image were transferred into Microsoft Excel for regression analysis.

## Fiber Treatments to Facilitate MFA Visualization

### Fiber Beating

Thirty oven dried (o.d.) grams of bleached kraft pulp fibers were run in the PFI mill for varying lengths of time. Of the 30-o.d. gram sample, 10 fibers were dyed with chlorazol black and photographed before introduction to the PFI mill. After 750, 3000, and 8000 revolutions, 3 of the original 10 fibers were located and photographed under DIC in order to evaluate fibril visualization.

### Fiber Stirring

Macerated kraft pulp fibers were stirred with a stir bar and magnetic stirrer for 2 hours in water (3 gm o.d./500mL).

**Fiber Cutting**

Fibers were placed onto a glass slide and a razor blade was used to dice the fibers into smaller pieces. After the fibers were cut, they were placed back into water, vigorously shaken and the fibers were then viewed using DIC.

**$CoCl_2$ + Ultrasound**

For treatment of both earlywood and latewood samples, about 200-300 dispersed fibers were put into a plastic weigh boat, excess water was removed by decanting, and a 5-10% solution of $CoCl_2$ (w/v) in water was poured into the weigh boat. Sufficient $CoCl_2$ solution was used so that all the fibers were either floating or immersed in the solution. The samples were left at room temperature overnight so that the $CoCl_2$ could diffuse into the fiber walls. Alternatively, comparable results were obtained after 3-4 hours at 50-60° C, although caution must be used not to evaporate all the solution and dry out the fibers. Earlywood fibers were exposed to the ultrasound in a bath (Branson 3200 at 47 kHz) for 15-20 minutes, while latewood fibers were exposed for 10-12 minutes [21]. Time may vary between different samples. Excessive fibrillation is possible using this treatment, and it is better to underfibrillate rather than to overfibrillate. Earlywood fibers were more difficult to fibrillate than latewood; thus, more time was required for earlywood.

**Rapid Freezing in Liquid Nitrogen**

Fibers at 50% consistency were immersed in liquid nitrogen for one minute. The fibers were then allowed to thaw at room temperature. Once thawed, the fibers were viewed under the DIC microscope.

**Freeze Drying**

Fibers at approximately 50% consistency were freeze dried overnight, dry mounted in Permount solution prior to viewing with DIC microscopy.

### Slow Freezing

Fibers at 50% consistency were placed on a glass microscope slide in a freezer at -5° C overnight. The fibers were then thawed and placed on a separate slide for viewing.

### NaOH Treatment

Approximately 0.5 grams of fibers at 50% consistency were immersed in 2M NaOH overnight.

## RESULTS AND DISCUSSION

When observing fibers from southern pine kraft pulps with a DIC microscope, it was noticed that microfibrils were clearly visible in a subset of fibers. These microfibrils were parallel and highly oriented suggesting that they were probably in the S2 layer (Figure 1). Focusing at different planes of the fiber secondary wall confirmed that the microfibrils were located in the S2 layer. The fact that cellulose microfibrils are visible in delignified fibers is readily explained by the theory behind differential interference contrast. DIC is a method to derive contrast from differences in the indices of refraction in unstained specimens [22]. In DIC microscopy, differences in the refractive index within the specimen phase shift the plane-polarized light and are transformed into detectable amplitude differences. The phase-distorted ray is sheared into two equally phased but spatially separated (perpendicular) rays by a Wollaston prism. Since DIC microscopes exploit phase differences between a specimen light ray and a reference ray they are interferometers. The ray pairs are recombined into a single vibrating plane in the analyzer such that constructive and destructive interferences produce light and dark areas enhancing the contrast of the image. A uniformly gray background is produced if no differences in the index of refraction exist within an area of a specimen. The advantage of DIC over phase contrast is that there is no bright diffraction halo. We reason that the microfibrils within the thick S2 layer of the fiber wall are visible with DIC because of

the difference in the index of refraction between the crystalline cellulose and the water that fills the voids left by lignin and hemicellulose after pulping or macerization.

When small wood samples are extensively delignified with peracetic acid, about 10-20% of the earlywood and 5-10% of the latewood fibers have cellulose microfibrils in the S2 layer that are directly visible by DIC microscopy. These initial observations suggested that DIC microscopy could be used to determine the MFA of delignified fibers. However, this method needed to be validated relative to other well-accepted methods and treatments needed to be identified that increased the number of fibers with visible microfibrils to minimize any potential sampling bias.

**Optimization of Fiber Preparation for DIC Microscopy**

A number of chemical and mechanical treatments were tested to increase the percentage of fibers that had visible microfibrils. Table I reports the sample treatments investigated and their qualitative effects on enhancing the visibility of microfibrils from macerated pine fibers by DIC microscopy. Overall the most beneficial treatments included some mild mechanical disruption. For example, the PFI mill produced beneficial results. At 750 revolutions, S1 fibrillation occurred. After 3000 revolutions, the S1 layer was apparently removed, and S2 fibrils appeared fibrillated. The S2 fibril angle was frequently enhanced after 3000 revolutions in the PFI mill. At 8000 revolutions, the S1 layer was completely removed, fibers appeared swollen, and excessive S2 fibrillation occurred. The fibrillation was so severe that it made viewing the MFA under DIC difficult. Choosing between fibrillated bundles and the true S2 MFA was often difficult. Stirring the fibers induced "ballooning" in localized areas of many fibers. This ballooning is likely to occur in places where defects such as microcompressions and dislocations had existed allowing water to become preferentially absorbed. Although microfibrils were visualized in these areas, due to the severity of swelling it was concluded that the MFA is likely altered, which would bias measurements.

The simplest and most effective treatment was to expose the fibers in a 5-10% solution of $CoCl_2$ to ultrasound waves while at room temperature. This treatment worked consistently well for increasing the number of fibers that had microfibrils visible by DIC microscopy. Earlywood fiber samples required about 15 minutes of ultrasound while latewood samples required less time, approximately 10 minutes. The cavitations created by the ultrasonic waves facilitated S2 microfibril identification through removal of the S1 layer and fibrillation in the S2 layer (Figure 2) [23]. It is hypothesized that the transition metal salt accelerates the disassociation of the bonds between microfibrils, allowing the fibril bundles or their voids to be observed with the DIC microscope. It should be noted that drying the fibers has a negative impact on this method, presumably by collapsing the space between the microfibrils that does not readily rehydrate.

**Comparison of DIC Microscopy to X-ray Diffraction**

To validate the utility of using DIC microscopy and image analysis for measuring fiber MFA, the results obtained from DIC microscopy were compared with those obtained from x-ray diffraction. From a set of loblolly pine wood samples visually screened for compression wood and uniform MFA using x-ray diffraction, three earlywood (E3, E7, and 7E3) and three latewood samples (L4, 20L2, and L5) of low (0-10°), medium (15-30°), and high (31-50°) MFA were selected. The MFA of these six wood samples was reconfirmed with x-ray diffraction in the tangential and radial positions. The samples were then gently delignified in peracetic acid, carefully rinsed in water, neutralized with 1M NaOH, and allowed to air dry overnight. The delignified earlywood samples were remeasured by x-ray diffraction and the differences in MFA from pulped and unpulped samples were negligible (Table II).

In a blind experiment, the MFA of the earlywood and latewood fibers from these six samples was measured by DIC microscopy and image analyses. Images of approximately forty different fibers

with clearly visible microfibrils were captured for each sample using a digital camera. In early work with the DIC method, measurement of 40 fibers gave similar means and standard deviations to 80 fibers. Thus, reducing the amount of fibers collected could save time; however, sample size optimization requires further study. Since the x-ray analysis was calibrated only to the tangential face of wood, it was desired to measure only the tangential face of the pulped fibers. However, the macerated earlywood fibers lay nearly exclusively with the radial wall up, most likely a result of the relatively inflexible bordered pits and somewhat thin secondary wall. Therefore, predominantly images of the radial wall of earlywood fibers were gathered. Latewood fiber samples had a greater percentage of tangential measurements, perhaps because the impact of the bordered pits was less in these thicker-walled fibers.

Table III summarizes the MFA measurements obtained from x-ray and DIC microscopy. Linear regression analysis showed that all six sets of data were highly correlated (Figure 3 and Table IV). X-ray tangential measurements and DIC combined show the highest degree of correlation between the two methods. This result should not be surprising since x-ray analysis was calibrated only in the tangential direction. It is important to note that in looking at tangential DIC and tangential x-ray, the data show nearly a unity slope and an excellent fit. The data are misleading, however. No tangential measurements were obtained from the E3 sample. The E3 sample gave the most deviant MFA results in this study, and by eliminating this sample set, the data appear to fit much better. In addition, tangential measurements may not be obtainable by DIC in some earlywood samples, like E3, for instance.

The regression line in Figure 3 suggests that DIC microscopy may have a tendency to slightly underestimate the MFA relative to x-ray diffraction. One potential cause of this lower estimate of MFA is that fibrils too close to the sides of the fiber had to be measured in some of the latewood tracheids. This problem was less frequent in earlywood measurements because thinner-

walled fibers tend to lay flatter on a slide. However, thick-walled latewood samples resist collapsing and make DIC measurements near the sides more likely to bias the mean of the population towards a slightly lower MFA. The slope of the latewood-only regression line is consistent with this explanation (Figure 3). In addition, looking at the data points in Figure 3, latewood is more frequently below the regression and the 45° lines in two of the three samples. Therefore, greater care seems to be necessary when deciding where to take fibril measurements in latewood.

**Fiber MFA Distribution Analysis by DIC and Polarization Confocal Microscopy**

Since DIC measures the MFA in single fibers, the distribution of MFAs was analyzed (Figure 4). Normal distributions where obtained from all the samples. The most deviant distribution appears to be E3, the earlywood with the lowest fibril angle. Perhaps the S2 and S1 layers were confused in a few measurements of this sample. This theory could explain why the MFA was overestimated in comparison to x-ray analysis. Furthermore, the E3 sample had by far the greatest range of MFAs of any of the other five samples. Since x-ray analysis does not provide single-fiber resolution, it cannot be used to compare the MFA distributions produced by DIC. Therefore, to validate whether the distributions of MFA in the fiber population are real and not an artifact of the DIC method, a sample was chosen from the initial x-ray study and MFA distributions and averages were compared to those obtained with polarization confocal microscopy congo red [12].

For this study, the high-angled latewood fiber L5 sample was chosen to compare MFA. This sample was chosen for two reasons: (1) to verify that the distributions obtained from DIC are not an artifact of the technique, and (2) to investigate whether DIC performed adequately with high MFA fibers. Since DIC produced two results in latewood that were 4° and 5° below x-ray diffraction, latewood samples were further investigated to attempt to explain these differences. One possibility is that, due to the variable nature of microfibrils both between and within individual fibers, the results

may also have been an artifact of insufficient sampling, n = 38 in both cases. For this comparison with polarization confocal microscopy congo red, approximately 250 fibers from the L5 sample were taken from the population. The DIC images of 152 fibers were captured and measured. After the images were captured the same subset of fibers were recovered and measured using the polarized confocal laser scanning microscope congo red method by H. Jang at Paprican in Vancouver, BC.

One hundred fibers were measured with the polarization confocal congo red method, and Figure 5 compares the results to those obtained from DIC. A mean of 48.3° ($\sigma$ = 5.3°) was obtained by DIC while the polarized confocal microscope measured a very similar MFA of 48.8° ($\sigma$ = 6.4°). A simple t-test showed no significant difference between the two means, and an f-test comparing the variances between the two methods indicated that they were different at a 95% but not at a 99% confidence. To a relatively high degree of confidence, the variance observed for the DIC technique was lower than the variance of polarization confocal microscopy. This slightly higher variance may be explained by greater reliance on image analysis of polarization confocal microscopy than with DIC microscopic method.

## CONCLUSIONS

DIC microscopy is a simple, accurate, and direct method for measuring the microfibril angle of single fibers. DIC microscopes adequately image the differences in the index of refraction of crystalline cellulose and the water that fills the voids left by lignin and hemicellulose after extensive pulping or maceration in the S2 layer of the fiber secondary wall; therefore, simple image analysis can be used to measure the MFA of thick-walled southern pine tracheids. The results of this DIC microscopy based method compare very favorably to those obtained from x-ray diffraction and polarization confocal congo red methods.

We have also shown that this method is suitable for measuring the MFA from hardwoods, including multiple poplar species, other softwoods, such as spruces, and nonwoody fibers.

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## REFERENCES

1. HARRIS, J., and MEYLAN, B.A. "The Influence of Microfibril Angle on Longitudinal and Tangential Shrinkage in *Pinus radiata*", *Holzforschung* 19: 144-153 (1965).

2. CAVE, I.D., and HUTT, L., "The Anisotropic Elasticity of the Plant Cell Wall", *Wood Sci. Tech.* 2: 268-278 (1968).

3. PAGE, D.H., "A Theory for the Tensile Strength of Paper", *TAPPI* 50(4): 674-681 (1969).

4. PAGE, D.H., and SETH, R.S., "Elastic Modulus of Paper", *TAPPI* 63(6): 113-116 (1980).

5. PAGE, D.H., SETH, R.S., and SOSZYNSKI R.M., "The Effect of Wood Species on the Edgewise Compressive Strength of Paper", *TAPPI J.* 94-96 (1986).

6. MEGRAW, R.A., LEAF, G., and BREMER, D., 'Longitudinal Shrinkage and Microfibril Angle in Loblolly Pine", "Microfibril Angle in Wood", University of Canterbury Press, Christchurch, NZ, (1998).

7. PAGE, D.H., EL-HOSSEINY, F., "The Mechanical Properties of Single Wood-Pulp Fibers. Part IV Fibril Angle and the Shape of the Stress-Strain Curve", J Pulp Paper Sci. 99-100 (1983).

8. MARK, R.E., and GILLIS, P.P., "The Relationship between Fiber Modulus and S2 Angle", *TAPPI J.* 56 (4) 164-167 (1993).

9. PAGE D.H., El-HOSSEINY, F., WINKLER, K., and LANCASTER, A.P.S., "Elastic Modulus of Single Wood Pulp Fibers", *TAPPI* 60 (4) 245-272 (1977).

10. CROSBY, C.M., and MARK, R.E., "Precise S2 Angle Determination in Pulp Fibers", *Svensk Papperstidning* nr (17); 636-642 (1974).

11. MEYLAN, B.A., Forest Prod. J. 17 (5) 51- (1967).

12. JANG, H.F., "Measurement of Fibril Angle in Wood Fibers with Polarization Confocal Microscopy", *J. Pulp Paper Sci.*, 24(7): 224-230 (1998).

13. ANAGNOST, S.E., HANNA, R.B and MARK, R.E., "Utilization of Soft Rot Cavity Formation as a Tool for Understanding the Relation Between Microfibril Angle and Mechanical Properties of Cellulosic Fibers", AMD 231: 43-63 (1999).

14. CAVE, I.D. "Theory of X-ray Diffraction method for the measurement of microfibril angle in wood", *For. Prod. J.* 16 (10): 37- (1966).

15. MEYLAN, B.A., " Measurement of Microfibril Angle by X-ray Diffraction", *For. Prod. J.* 17 (5): 51-58 (1967).

16. EL-OSTA, M., LOFTY, M., KELLOGG, R.M., FOSCHI, R.O., and BUTTERS, R.G., "A Direct X-ray Technique for Measuring Microfibril Angle", *Wood Fibre* 5(2): 118- (1973).

17. EVANS, R, STUART, S, and VAN DER TOUW, J., "Microfibril Angle Scanning of Increment Cores by X-ray Diffractometry", *Appita J.* 49(6); 411-414 (1996).

18. PAGE, D.H, "A Method for Determining the Fibrillar Angle in Wood Tracheids", *J. Microscopy* 90(2): 137-143 (1969).

19. BATCHELOR, W., CONN, A., and PARKER, I., "Measuring the Fibril Angle of Fibers Using Confocal Microscopy", *Appita*, 50(5): 377-379 (1997).

20. El-HOSSEINY, F., and PAGE, D.H., "The Measurement of Fibril Angle of Wood Fibers Using Polarized Light", *Wood and Fiber Sci.* 5(3): 208-214 (1973).

21. WANG, H.H., DRUMMOND, J., HUNT, K., and WATSON, P., "An Improved Fibril Angle Measurement Method for Wood Fibers", Paprican Pulp and Paper Report, PPR 1268, February 1997.

22. PLUTA, M., "Advanced Light Microscopy Methods, Specialized Methods" , Volume 2, Elsevier, Amsterdam.

23. IWASAKI, T., LINDBERG, B., and MEIER, H., "The Effect of Ultrasonic Treatment on Individual Wood Fibers," *Svensk Papperstidning*, 65 (20): (1962).

Table I
**Effect of Fiber Treatments on Enhancing the Percentage of Fibers with Microfibrils Visible by DIC Microscopy**

| Treatment | S2 MFA Visualization |
|---|---|
| Congo red staining | ± |
| PFI mill | ++ |
| NaOH (fiber swelling) | ± |
| Ultrasonic treatment w/ $CoCl_2$ | + + + |
| Fiber cutting | + |
| Liquid nitrogen | ± |
| Freeze drying | - |
| Slow freezing (- 5° C) | + |
| Stirring | +, ± |

+++ = Very Positive
++ = Positive
+ = Sporadic
± = Neutral
- = Negative

Table II

**MFA of Unmacerated and Macerated Wood Chips from X-ray Diffraction**

| Sample | Unmacerated | Macerated |
|---|---|---|
| Late 1 (L4) | 6 | n.d.* |
| Late 2 (20L2) | 51 | n.d. |
| Late 3 (L5) | 33 | n.d. |
| Early 1(E3) | 6 | 4 |
| Early 2 (E7) | 29 | 30 |
| Early 3 (7E3) | 42 | 44 |

*not determined

Table III: **Summary of DIC and X-ray MFA Measurements**

| Sample | MFA (X-ray analysis) | MFA (DIC) | DIC confidence interval (95%) |
|---|---|---|---|
| E3 | 5.33° | 13.4° | ± 2.3° |
| 7E3 | 42° | 44.0° | ± 1.4° |
| E7 | 29.67° | 30.0° | ± 1.5° |
| L4 | 6.25° | 5.6° | ± 1.4° |
| 20L2 | 33° | 27.9° | ± 2.8° |
| L5 | 51° | 47.0° | ± 1.6° |

**Table IV: Linear Regression Analysis Between X-ray and DIC Methods: Comparison of Radial and Tangential Measurements**

| Regression | X-ray Tangential | X-ray Radial | X-ray Combined | DIC Combined | DIC Radial | DIC Tangential |
|---|---|---|---|---|---|---|
| X-ray Tangential | | 1.04, 0.973 | 1.03, 0.977 | 0.949, 0.954 | 0.95, 0.953 | 0.946, 0.975 |
| X-ray Radial | | | 0.99, 1.0 | 0.909, 0.94 | 0.911, 0.943 | 0.906, 0.951 |
| X-ray Combined | | | | 0.911, 0.939 | 0.913, 0.942 | 0.908, 0.955 |
| DIC Combined | | | | | 1.0, 0.999 | 1.0, 0.992 |
| DIC Radial | | | | | | 1.0, 0.987 |
| DIC Tangential | | | | | | |

**Figure Legends**

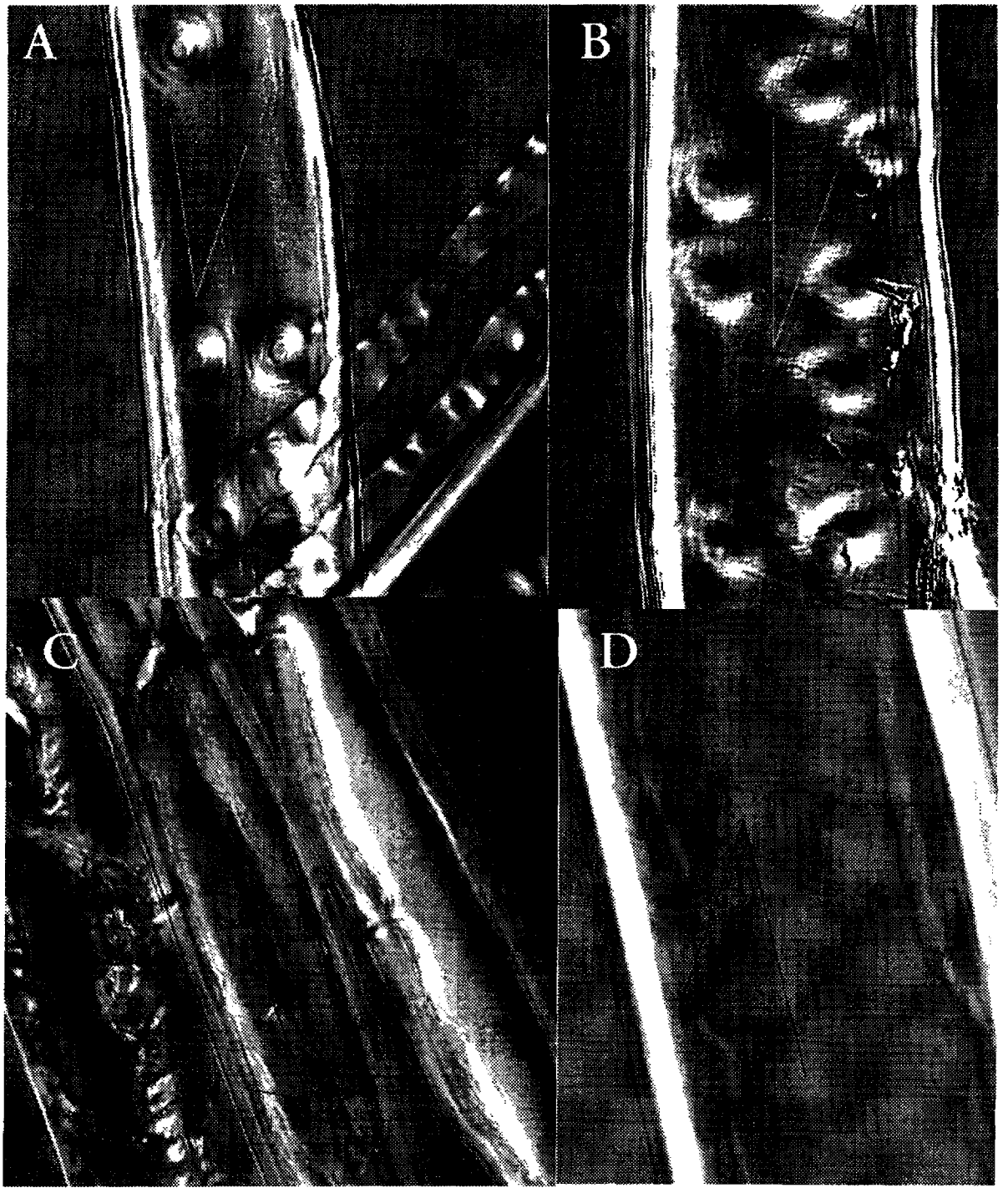
Figure 1. Representative DIC images with visible microfibrils in the S2 layer of earlywood and latewood tracheids, Earlywood (A) 20X objective and (B) 40X objective Latewood (C) 20X objective and (D) 40X objective. The line represents the angle of cellulose microfibrils relative to the longitudinal axis of the cell.

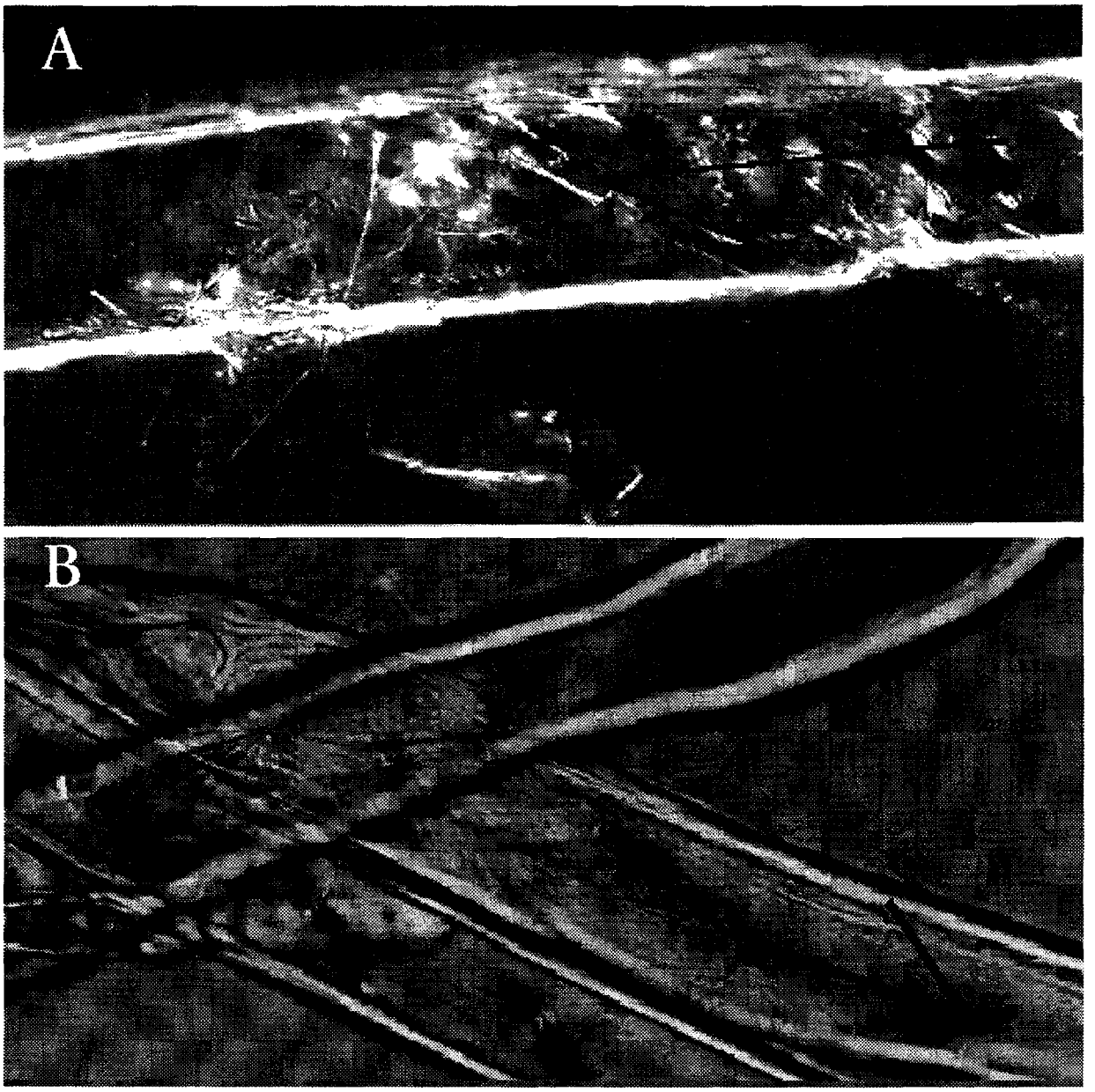
Figure 2. Representative DIC images after $CoCl_2$ and ultrasound treatment. Ultrasound treatments were 15 minutes for earlywood and 10 minutes for latewood. The line represents the angle of cellulose microfibrils relative to the longitudinal axis of the cell.

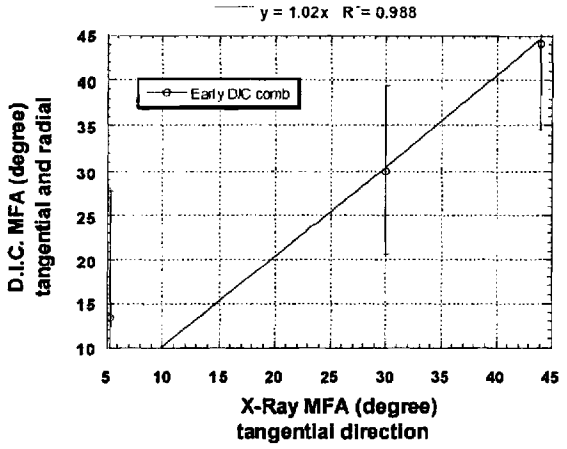
Figure 3. Linear regression analysis of earlywood (A), latewood (B) and combined earlywood and latewood (C)

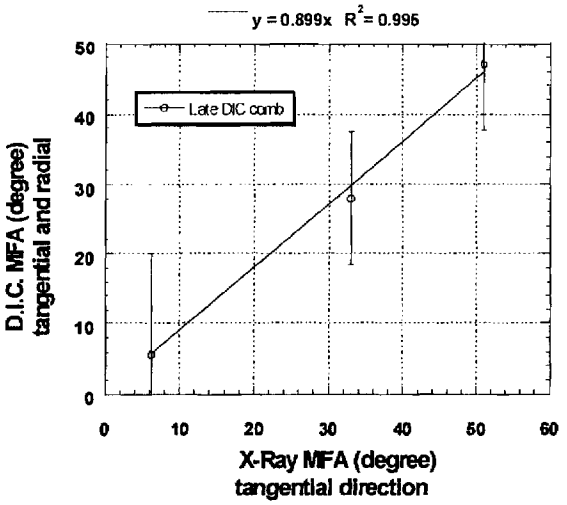
Figure 4. The range and distribution of single-fiber MFAs in small wood samples measured by DIC microscopy and image analysis.

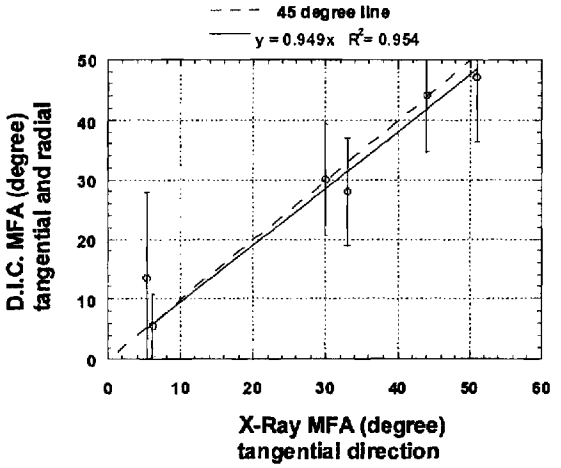
Figure 5. Comparison of the distribution of DIC and polarization confocal microscopy congo red.

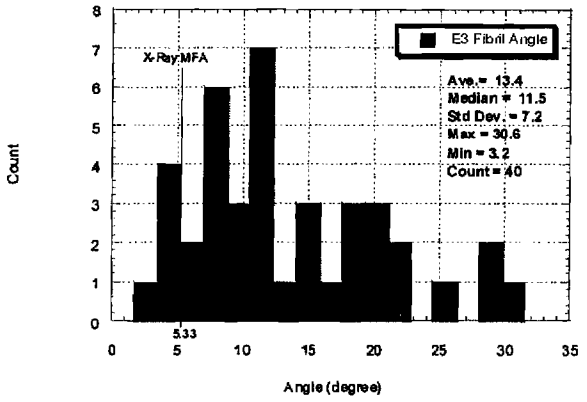

A
B
C
D

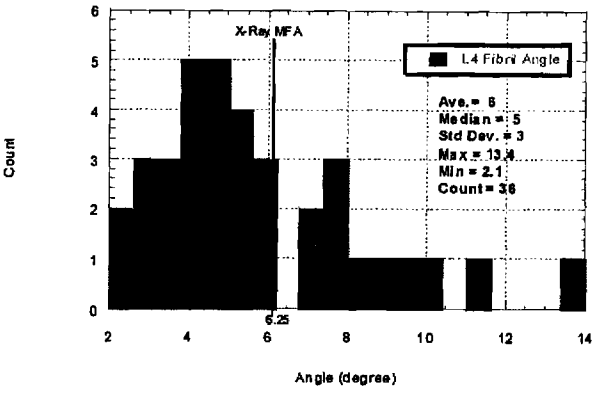

A
B


y = 1.02x R²= 0.988
Early DIC comb
D.I.C. MFA (degree)
tangential and radial
X-Ray MFA (degree)
tangential direction

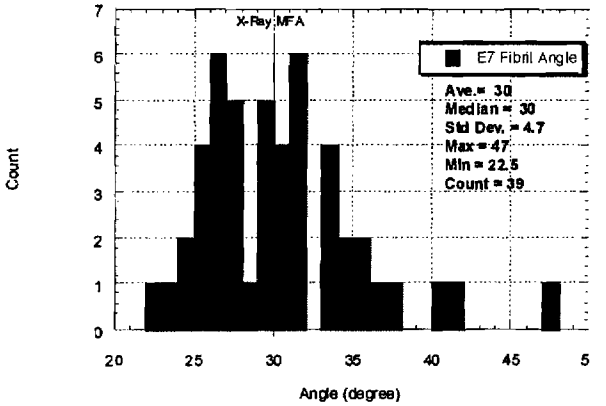

y = 0.899x R²= 0.995
Late DIC comb
D.I.C. MFA (degree)
tangential and radial
X-Ray MFA (degree)
tangential direction

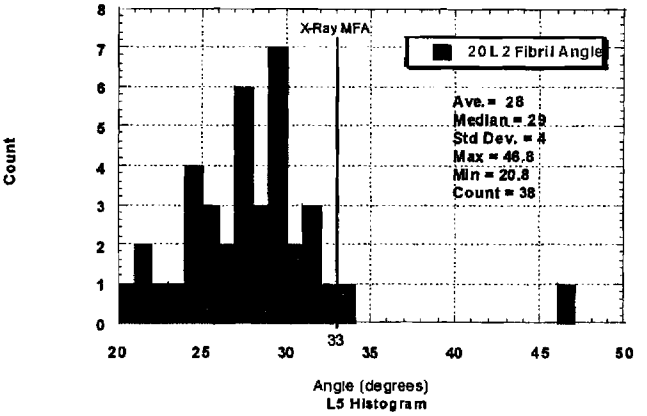

45 degree line
y = 0.949x R²= 0.954
D.I.C. MFA (degree)
tangential and radial
X-Ray MFA (degree)
tangential direction



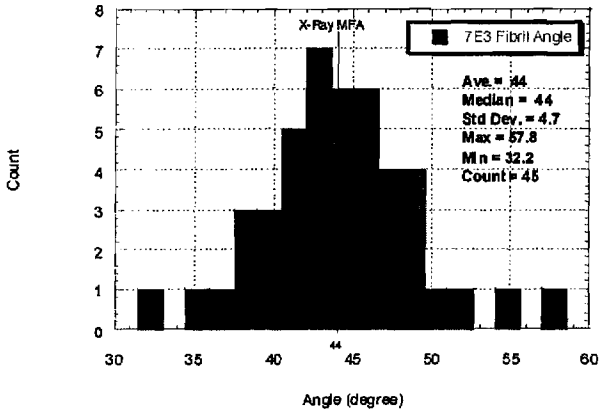



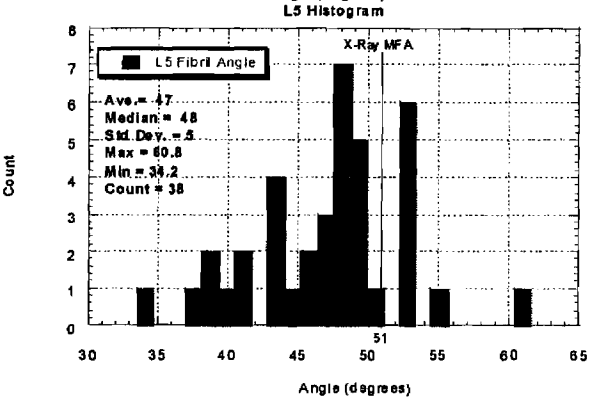

20 L2 Histogram

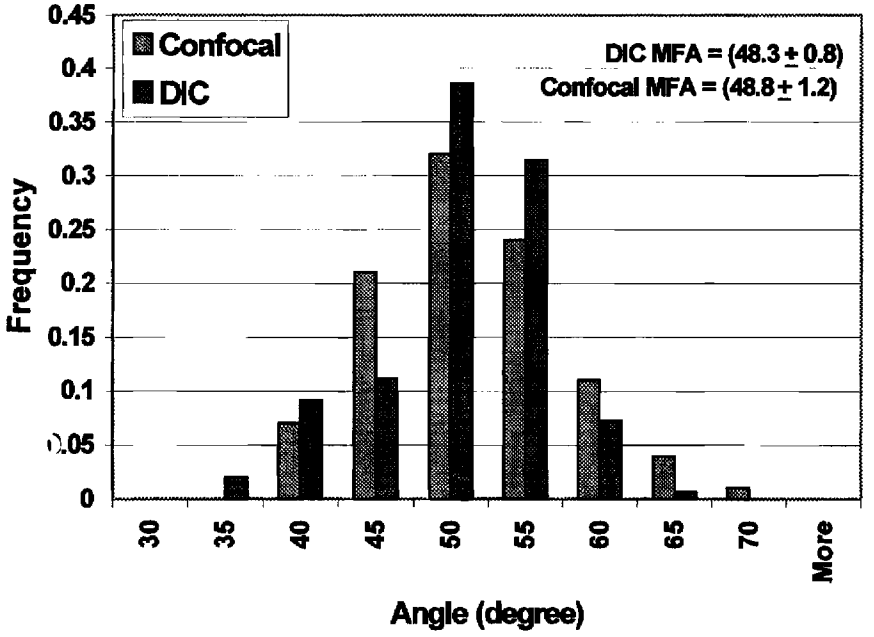



L5 Histogram




Confocal
DIC
DIC MFA = (48.3 ± 0.8)
Confocal MFA = (48.8 ± 1.2)
Frequency
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
30
35
40
45
50
55
60
65
70
More
Angle (degree)